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Light & Electron Microscopy of Dogs' Lungs after Long-Term Exposure to Ozone

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Final Report

STUDY OF LUNGS FROM BEAGLE DOGS FOR SUBELECTRON AND ELECTRON MICROSCOPY FOLLOWING EXPOSURE TO OZONE

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16. Abstracts Female beagle dogs were exposed for about 18 months to 1 ppm of ozone in air for either 8, 16, or 24 hours per day and to 2 or 3 ppm for 8 hours a day. The earliest evidence of response was the appearance of macrophages in and around the respiratory bronchiolar-ductal region and in the adjacent alveoli. The macrophages increased with the "dosage," the macimum concentrations being associated with 3 ppm for 8 hours per day. Although fibrous tissue elements were only rarely deposited at the lowest dosage, deposition became evident in occasional alveolar ducts following exposure for 16 hours daily to 1 ppm, and increased as the concentration of ozone rose. Features more apparent at the higher concentrations were thickening of the terminal and respiratory bronchiolar walls and their infiltration by lymphocytes and some plasma cells and "fibroblasts" that formed peribronchiolar collars of cells. Strands of connective tissue which contributed to the narrow alveolar openings in the respiratory bronchioles and in the alveolar ducts, extended into the attached alveolar walls. Bronchiolar walls, thickened by infiltration with cells and deposition of connective tissue, reduced the caliber of small airways.

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LIGHT AND ELECTRON MICROSCOPY OF DOGS' LUNGS AFTER LONG-TERM EXPOSURE TO OZONE*

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The universal presence of solar photochemical energy induces reactions in smoggy atmospheres between oxides of nitrogen (NO_x) and oxygen (O_2). As a result, both of the interdependent toxic gases, nitrogen dioxide (NO_2) and ozone (O_3), are produced and so contaminate the atmospheric environment during the cycle of each day.

Structural changes in animals exposed for long periods to low levels of nitrogen dioxide (NO_2) have been extensively examined (1-4). The pathology of animals similarly exposed to ozone (O_3) has seldom been studied. Generally, the pathology in animals and man has been described following exposures that were likely to induce edema, hemorrhage, and inflammation (5-8). Even fewer ultrastructural observations have been made (9). On the other hand, considerable information is available about the biochemical effects of O_3 (5).

The current studies, therefore, were designed to allow animals to survive for at least 18 months with daily exposure to air containing from 1 to 3 parts per million (ppm) of O_3. In a similarly prolonged study, Stockinger et al. examined tissues from animals treated to induce chronic disease (10), and Gross et al. found evidence of a "pre-emphysematous" condition in some of the material (11). This report describes microscopic and ultrastructural morphologic effects in dogs' lungs that appear to have resulted from exposure to subacute levels of O_3.

The protocol for exposure and in vivo studies of the dogs will be the subject of another report (Lewis et al., unpublished). Essentially, 48 female beagles were assigned randomly to 6 exposure chambers, each to contain 8 animals, thus forming 6 groups. Three groups were exposed to 1 ppm of ozone--Group A for 8 hours per day, Group B for 16 hours daily, and Group C for 24 hours. Group D was exposed to 2 ppm and Group E to 3 ppm, both for 8 hours per day. (Group B had been exposed to 3 ppm for 16 hours for one day, with acute manifestations of induced illness). Therefore, the animals were withdrawn and reintroduced the following day into a chamber containing only 1 ppm. Group F, a control, was kept in a chamber containing filtered air only.

Physiologic studies were performed at intervals during the 544 days of the experiment (unpublished data). The dogs were sacrificed in random order, using an overdose of sodium pentobarbital. Gross observations of the lungs were not made. Immediately after the trachea was clamped off, the thorax was opened, and the right apical lobe was removed and injected with gluteraldehyde through the bronchial stump. The lobe was then immersed in the same solution. The entire left lung was filled with 10% formalin. After fixation, representative blocks were excised from the diaphragmatic, middle, and apical lobes.

At the time of sacrifice, additional tissue was obtained immediately for electron microscopy by placing a hemostat across a portion of the right lung and removing the distal tissue. A blunt needle on a syringe, filled with 1% OsO_4 buffered to a pH of 7.4, was inserted into the largest airway on the cut surface. A string was tied around the tissue

close to the cut surface to include the needle, and the string was pulled tight. The severed piece of lung was then filled just to capacity with the fixative. The needle was removed and the piece of lung was immersed in an aliquot of the same fixative for approximately 45 minutes. The tissue was then cut into 1-mm slices through the longitudinal axis with a sharp razor blade, and the slices were placed in 70% ethanol.

The remainder of the right apical lobe was fixed in 2% gluteraldehyde brought to a pH of 7.3 with cacodylate buffer. The remainder of the lung was reserved for bacteriologic culture and biochemical studies. All tissue specimens were then shipped from the Air Pollution Control Office in Cincinnati (where the animals were exposed and sacrificed) to Stanford Research Institute.

The formalin-fixed tissue blocks were paraffin-embedded, and sections were prepared and stained with hematoxylin and eosin and also with analine blue and orange G for collagen. The gluteraldehyde-fixed and OsO_4-fixed tissue specimens were further prepared and embedded according to the procedure described previously (12). After polymerization of the embedding medium, the blocks were trimmed and 1-micron sections were cut for light microscopy. Precise areas were selected and the block was again trimmed for electron microscopy. Sections were placed on uncoated nickel grids, stained with lead citrate and uranyl acetate, and examined in a Philips 200 electron microscope.

A statistical evaluation of macrophages in the bronchiolar-ductal area was made for control and experimental dogs. Random fields along the terminal airways were counted, using a magnification of 500x. One edge of the field was placed just beneath the wall of the airway; the remainder of the field extended into the alveoli. Twenty-five fields were examined in the control and in each of the experimental dogs, and the average number of macrophages per field was calculated.

Results

The 69 blocks prepared from 23 animals for formalin-fixation were a random sampling of the 6 groups of beagles. Changes in the alveolar parenchyma peripheral to the bronchioles and alveolar ducts could not be assessed due to variability in end-pressures used for filling the lungs with formalin and, thus, also to differences in distention and attenuation of alveolar walls.

Normal Dog Lung

The long, muscular bronchiole of the normal dog lung (Group F) is shown in sagittal section (Figure 1) from its proximal to a distal portion, at which point the respiratory bronchiole and the alveolar duct give rise to the alveoli of the parenchyma. Noteworthy characteristics of this normal bronchiolar area are the uniformity of the surrounding alveolar network and its relative freedom from extraneous cells. The alveoli are attached simply to the muscular bronchiolar and ductal

framework. Only a few nuclei, sometimes in short chains, are visible on the alveolar sides of the airway walls. The alveolar septa themselves have a uniform thickness throughout.

For electron microscopy, the complexity of the terminal airways of the dog required precise handling of tissue to obtain useful information from the small samples. Mucus-producing cells of the proximal portion are interspersed among the richly ciliated columnar epithelium (Figure 2). As the bronchiole extends peripherally, the goblet cell population gives way to nonciliated cells (Figure 3) and the number of cilia per cell also decreases (Figures 3 and 8), but the microvilli covering the apices of these cells remain (Figure 8). The proportion of nonciliated to ciliated cells continues to increase so that the muscular rings at the proximal end of the respiratory bronchiole are covered only by nonciliated, cuboidal cells. These contain substantial amounts of glycogen (Figures 4 and 8). Distally, the muscular rings become thinner and the cuboidal cells that cap them (Figures 5 and 6) give way progressively to a squamous type (Figure 7).

Lungs Exposed to Ozone

The terminal airway with its immediately adjacent alveoli was the anatomic level most obviously affected by ozone. When compared with controls, minimal fibrosis was observed very occasionally and randomly in the periphery of an alveolar duct in Group A animals. Also, a few "extra" macrophages were seen in the central alveoli of these animals. Otherwise, the lungs were quite normal.

In lungs of Group B, occasional fibrous strands were deposited in some alveolar openings in the respiratory bronchioles and alveolar ducts. It became apparent, also, that the number of macrophages in the lungs was a function of the degree of exposure to ozone (Figure 9). [Of special interest is the lower number of macrophages in animals having been exposed to 1 ppm for 24 hours per day than in those with the same "dose" (concentration x time), i. e., exposed to 3 ppm for 8 hours per day.]

Figure 10 is from a dog that was exposed continuously to 1 ppm (Group C). It depicts a typical respiratory bronchiole and alveolar duct with associated alveoli, revealing relatively broad strands of connective tissue extending from the airways into alveolar walls and, sometimes, linking together groups of alveoli in the ductal areas. Also, connective tissue contributes to the thickness of the ductal walls on the alveolar side. Alveolar openings in the respiratory bronchiolar and ductal walls are distorted by connective tissue strands. The layer of fibroblast-like cells suggests active deposition of collagen. Epithelial hyperplasia is seen sporadically in the bronchiolar-ductal zone. Isolated foci of fibrotic distortion of distal parenchymatous alveoli, and mononuclear cellularity of the septa, presumably due to O_3, are seen in Figure 11. There is no evidence of an associated inflammatory process.

The increasing evidence of response to ozone, as the duration of exposure to 1 ppm was prolonged, was exaggerated in dogs breathing 2 ppm for 8 hours per day (Group D), as seen in Figure 12. The terminal airways clearly have thicker walls, and mononuclear cells aggregate on their parenchymal aspects, involving also the attached alveolar walls. The fibrosis permeating these structures appears to be associated with contraction, shortening and distortion of the terminal airways. Stratification of squamous epithelial cells was noted in the lumenal walls, together with a tendency for the cuboidal and columnar bronchiolar epithelium to become flatter.

In animals exposed daily for 8 hours to 3 ppm (Group E), the lesions described above were more frequent and usually more advanced (Figures 13-15). Cuboidal epithelium tended to become squamous (Figures 16 and 23), and the glycogen content of these cells was reduced in comparison with those in controls (cf 4 and 8 with 16 and 23). The low cuboidal cells of exposed animals have numerous microvilli projecting from their lateral surfaces into the intracellular space (Figure 23). One or more stacks of Golgi cisternae are observed and there is a substantial increase in the amount of rough endoplasmic reticulum. Several lysosomal-like inclusions, together with a moderate population of mitochondria, are present in the cytoplasm. The interface between the cells and the basement membrane is relatively simple with few undulations. However, as squamous stratification develops (Figure 24), the homogenous material in the basement membrane area becomes thickened and its interface with the epithelial cells becomes irregular. In animals exposed to 3 ppm ozone, such characteristic areas of squamous metaplasia composed of two or more layers of cells (Figures 17, 18, and 24) alternate frequently with groups of low cuboidal epithelium.

Fibrosis was fairly universal among the bronchiolar-ductal units and the immediately contiguous alveoli they serve. Figures 13, 14 and 15 reveal such fibrosis in the terminal airways and the associated aggregations of macrophages. Thickened bronchiolar walls (characteristic of this group of animals), accumulations of peribronchiolar--as well as "free"--cells in the bronchiole-associated region, and fibrotic thickening of adjacent alveolar septa are visible. Invading and proliferating lymphocytes, plasma cells and fibroblasts form a collar of mononuclear cells on the parenchymal aspect of the terminal airways, as seen under higher magnification in Figures 19 and 20.

Thus, three types of bronchiolar epithelial response to ozone were observed. One was "activation" of columnar epithelial cells, represented in Figure 21 as an extraordinarily high proportion of mucus-forming cells for this level of the airway. Mucus-producing cells extended peripherally, being encountered well beyond the proximal end of the respiratory bronchioles. A second type is the squamous metaplasia that occurs in areas contiguous to cuboidal epithelium (Figures 17 and 18). Intercellular bridging is characteristic of such stratified squamous epithelium. Squamous metaplastic cells exhibit prominent desmosomes when examined ultramicroscopically (Figures 23 and 25). Bundles of tonofila-

nents extend from the desmosomes and continue through the cytoplasm. Many microvilli protrude from the surfaces of the squamous metaplastic cells into the intercellular spaces. Additional cytoplasmic features were the rather sparse population of mitochondria, the moderate number of free ribosomes, and the paucity of rough endoplasmic reticulum. In some cells, small vacuoles were distributed preferentially in the superficial cytoplasm (Figure 26) and an occasional multivesicular body was also observed (Figure 23). Figure 22 shows a third type, an epithelial focus of excessive hyperplasia within a field of cuboidal cells.

There was variability among exposed animals in individual groups, so that certain lungs appeared relatively little affected compared to others in the group. Also, an occasional animal, expecially among those exposed to 3 ppm (Group E), harbored isolated foci of acute inflammation, suggesting recent infection in addition to the ozone effect.

Discussion

The lesion induced by exposing dogs to nonedema-inducing and noninflammatory concentrations of ozone was most easily recognizable in the bronchioles, respiratory bronchioles, and alveolar ducts. Changes were barely noticeable at the lowest dose but were more evident in animals exposed for 16 hours daily to 1 ppm, and were most advanced in those exposed to 3 ppm for 8 hours daily. In the last group, squamous metaplasia and stratification of cuboidal cells developed in the bronchiolar epithelium. Thickening of the muscular bronchiolar walls was due in part to elements of connective tissue. In the region of the alveolar ducts, strands of connective tissue reduced the alveolar openings, appearing to constrict (if not obstruct) them. Also, some distortion and contraction of the terminal airways are suggested. In response to the ozone, mononuclear cells were found to congregate on the bronchiolar walls and to occupy space in the attached alveoli. Macrophages also were attracted to the area and were found in alveoli adjacent to the terminal airways, as well as within the lumens of the airways.

Similarities and differences in effects from other air pollutants, such as NO_2, cannot be assessed with confidence until comparative studies have been carried out in the same species. Nevertheless, the tendency for deposition of connective tissue in the ductal and associated alveolar walls of dogs recalls similar changes in rats that had been exposed to low levels of ozone over long periods of time (unpublished data). Lungs of rats that had been exposed to NO_2 alone at equivalent concentrations have been described (1-4). The focal point of the lesion in rat lungs induced by ozone tends to be slightly more distal than that caused by NO_2, and fibroblastic activity is more evident than that induced with NO_2. Also, the terminal bronchiolar epithelium of the ozone-exposed rat appears to be somewhat less hypertrophic than that of the NO_2-exposed rat, although a meaningful comparison would require a quantitative basis for determining some form of pharmacologic equivalence.

In any event, it is clear from observations in both the dog and in the rat that ozone is more injurious than NO_2 in terms of atmospheric concentration. Thus, the question about the relative effects of the associated gases, ozone and NO_2, as they occur realistically in a mixture in polluted air, compared to the effects of either gas alone, is germane.

The subcellular responses of pulmonary cells to ozone, as observed earlier with NO_2 in rats (13-15), are of special interest. Intracytoplasmic bodies in the bronchiolar epithelium of control dogs were exaggerated in those that had been exposed to non-necrotizing levels of ozone (16). Interpretation is wanting, which suggests the need to explore the intracellular biochemical events responsible for the changes.

Although there are similarities in the response of pulmonary tissue of the airways to NO_2 and to ozone, the chemical evidence does not favor identical peroxidative reactions (17). The apparent differences in fibroblastic response may indicate some degree of specificity in biological reaction toward the respective gases. Also, the difference in the anatomic level of the respiratory airway at which the response is greatest suggests differences in solubility and aerodynamics between the individual gases.

The squamous metaplasia of the bronchiolar epithelium is of particular interest because it occurs characteristically in animals deficient in vitamin A (18, 19). The vitamin is readily oxidizable.

Although the dog is relatively resistant to ozone, the mounting evidence suggests that high atmospheric concentrations of the gas may contribute to pulmonary disease. Its inevitable appearance--rapidly in the wake of NO_2--may act to enhance the injurious characteristics of photochemically induced smog and contribute to endemic respiratory disability in susceptible individuals, as well as to be a factor in chronic obstructive pulmonary disease.

Summary

Female beagle dogs were exposed for about 18 months to 1 ppm of ozone in air for either 8, 16, or 24 hours per day and to 2 or 3 ppm for 8 hours a day. The earliest evidence of response was the appearance of macrophages in and around the respiratory bronchiolar-ductal region and in the adjacent alveoli. The macrophages increased with the "dosage," the maximum concentrations being associated with 3 ppm for 8 hours per day. Although fibrous tissue elements were only rarely deposited at the lowest dosage, deposition became evident in occasional alveolar ducts following exposure for 16 hours daily to 1 ppm, and increased as the concentration of ozone rose.

In addition to aggregation of macrophages and deposition of connective tissue fibers in the ducts, features more apparent at the higher concentrations were thickening of the terminal and respiratory bronchiolar walls and their infiltration, mainly on the alveolar side, by lymphocytes

and some plasma cells and "fibroblasts" that formed peribronchiolar collars of cells. Strands of connective tissue which contributed to the narrow alveolar openings in the respiratory bronchioles and in the alveolar ducts, extended into the attached alveolar walls. Bronchiolar walls, thickened by infiltration with cells and deposition of connective tissue, reduced the caliber of small airways. Bronchiolar epithelial changes in dogs exposed to the higher concentrations were represented by a high proportion of mucus-forming cells (and their peripheral extention) and squamous metaplasia of columnar and cuboidal cells with occasional islands of epithelial hyperplasia. Metabolic effects of ozone are reflected in the ultrastructure of pulmonary cells in which are revealed the alteration of novel, preexisting, intracytoplasmic bodies (16).

Together with its associated photochemical reaction product, NO_2, ozone may contribute to pulmonary disease in an exposed susceptible population.

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EXPLANATION OF FIGURES

Figure 1 — Composite of three contiguous fields demonstrating normal terminal bronchiole, respiratory bronchiole, alveolar duct, and adjacent alveoli. Alveolar clefts in the respiratory bronchiolar and ductal walls are clearly patent. Free cells are rare. 80x H&E

Figure 2 — Tissue taken from a normal bronchiole an order of size larger than the terminal bronchiole. The pseudo-stratified columnar epithelium has a rich bed of cilia and large goblet cells are seen. 1,250x (Toluidine blue)

Figure 3 — Nonciliated cells (NC) increase in frequency from the proximal to the distal end of the normal terminal bronchiole. Mucus-containing cells usually are not found in the epithelium of the terminal bronchiole. 1,250x (Toluidine blue)

Figure 4 — At the distal end of the normal terminal bronchiole and most of the respiratory bronchiole, cuboidal, nonciliated epithelium lines the airway. The large, pale, homogenous intracytoplasmic areas contain glycogen (G). Smooth muscle bundles (SM) underlie the epithelium. 1,250x (Toluidine blue)

Figures 5 & 6 — The lumenal surfaces of the muscle bundles in the normal respiratory bronchioles are covered distally by simple cuboidal epithelium. 1,250x (Toluidine blue)

Figure 7 — In the normal alveolar ducts, the smooth muscle bundles may be covered only with thin cytoplasmic extensions resembling Type I alveolar cells. 1,250x (Toluidine blue)

Figure 8 — Electron micrograph showing normal epithelial cytology of ciliated and nonciliated cells of the terminal bronchiole. Microvilli (MV) as well as cilia (C) protrude from the apical surface. The ciliated cell at the top is proximal to the lower one and possesses more cilia. Basal granules can be seen in the apical cytoplasm. The nonciliated cells contain large deposits of glycogen (G). 7,4000x

Figure 9 — (Graph) Average number of macrophages per field along peripheral airways in control and experimental lungs.

Figure 10	Early changes in a dog exposed continuously to 1 ppm. In the alveolar duct and associated alveoli, strands of connective tissue increase the thickness of some alveolar walls. Alveolar macrophages are present. 80x H&E
Figure 11	This lung, after daily exposure to 1 ppm for 16 hours, reveals the unusual event of early parenchymatous fibrosis at a distance from a terminal airway. 80x H&E
Figure 12	Following exposure to 2 ppm for 8 hours daily, this bronchiole and alveolar duct reveal narrowing of alveolar orifices due to deposition of connective tissue along the muscular walls and to the tendency for such tissue to contract. Cellularity in affected lungs characterizes the peribronchiolar and periductal tissue and extends into attached alveolar walls. 80x H&E
Figure 13	Lesion similar to that in Figure 12 after daily exposure for 8 hours to 3 ppm. 80x H&E
Figures 14 & 15	Additional examples of lesion seen in Figure 1[illegible]. 125x TB(14) and 80x H&E(15)
Figure 16	Squamous, rather than cuboidal, epithelium lines much of the respiratory bronchiole in an experimental animal, and indicates a change from normal (see Figures 4 and 5). The increased width of interstitial space (arrows) underlying the epithelium is notable. 55x (Toluidine blue)
Figures 17 & 18	In focal areas, squamous metaplasia is formed in exposed lungs by two or more layers of cells held together by intercellular bridges. 1,250x (Toluidine blue)
Figure 19	Cellularity is often prominent in the terminal airways of exposed dogs. Here, smooth muscle bundles (SM) are surrounded by large numbers of cells after exposure to 3 ppm. 555x (Toluidine blue)
Figure 20	Proliferation of squamous cells lining the muscle bundles that encircle the respiratory bronchioles and alveolar ducts and aggregation of mononuclear cells between the duct and its adjacent alveoli. Exposure was to 3 ppm for 8 hours daily. 200x H&E
Figure 21	Increased number and activity of mucus-forming cells in the terminal bronchiole of a dog exposed to 3 ppm for 8 hours daily. 200x H&E

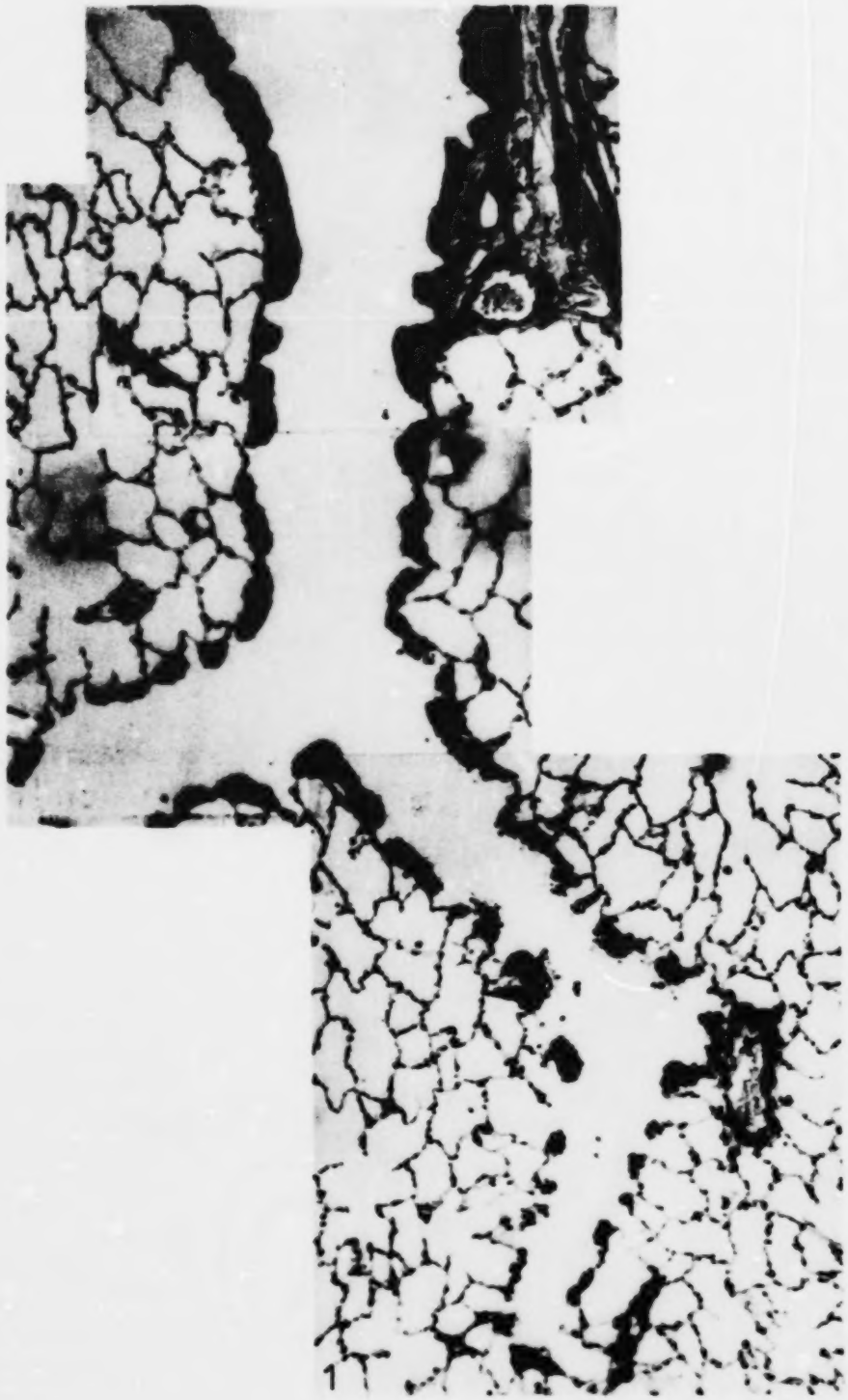
Figure 22 Focal pile of stratified epithelium in area of normally cuboidal cells of terminal bronchiole after exposure to 3 ppm for 8 hours daily. 504x H&E

Figure 23 Electron micrograph of a "low cuboidal" cell from an area similar to that in Figure 16. Numerous microvilli protrude from the lateral plasma membranes into the intercellular space. A moderate population of mitochondria, together with several lysosmal-like bodies (L), are present in the cytoplasm. A Golgi complex (Go) is to the right of the nucleus. 40,250x

Figure 24 A section through squamous metaplasia. The homogenous material (HM) in the interstitial space underlying the epithelium is much thicker than normal and the contour of the basal plasma membranes is very irregular. 22,680x

Figure 25 Another example of squamous metaplasia at higher magnification. Numerous desmosomes are observed attaching cells to each other. Tonofibrils are seen within the cytoplasm (arrows). 14,000x

Figure 26 Stratified squamous cell containing numerous small vesicles in the peripheral cytoplasm. 31,200x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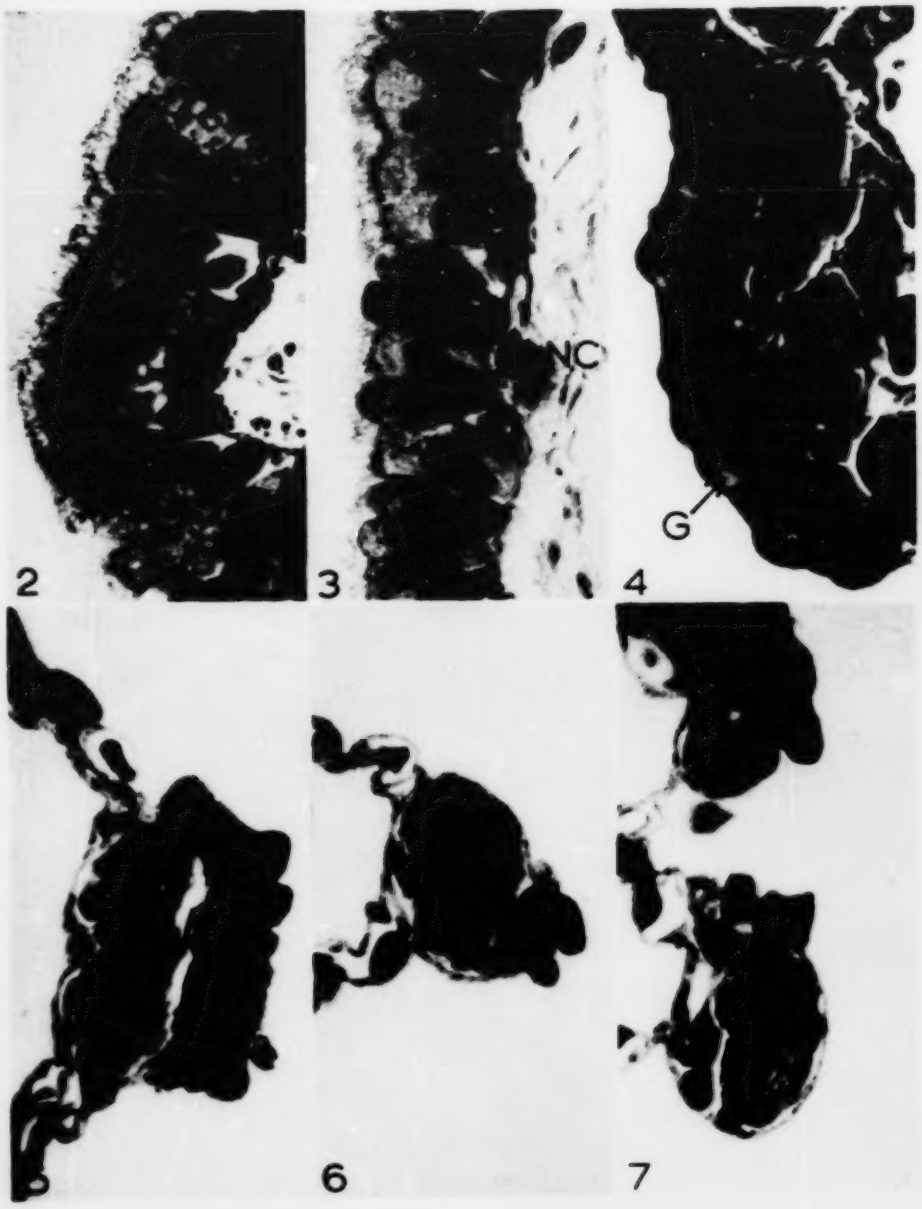
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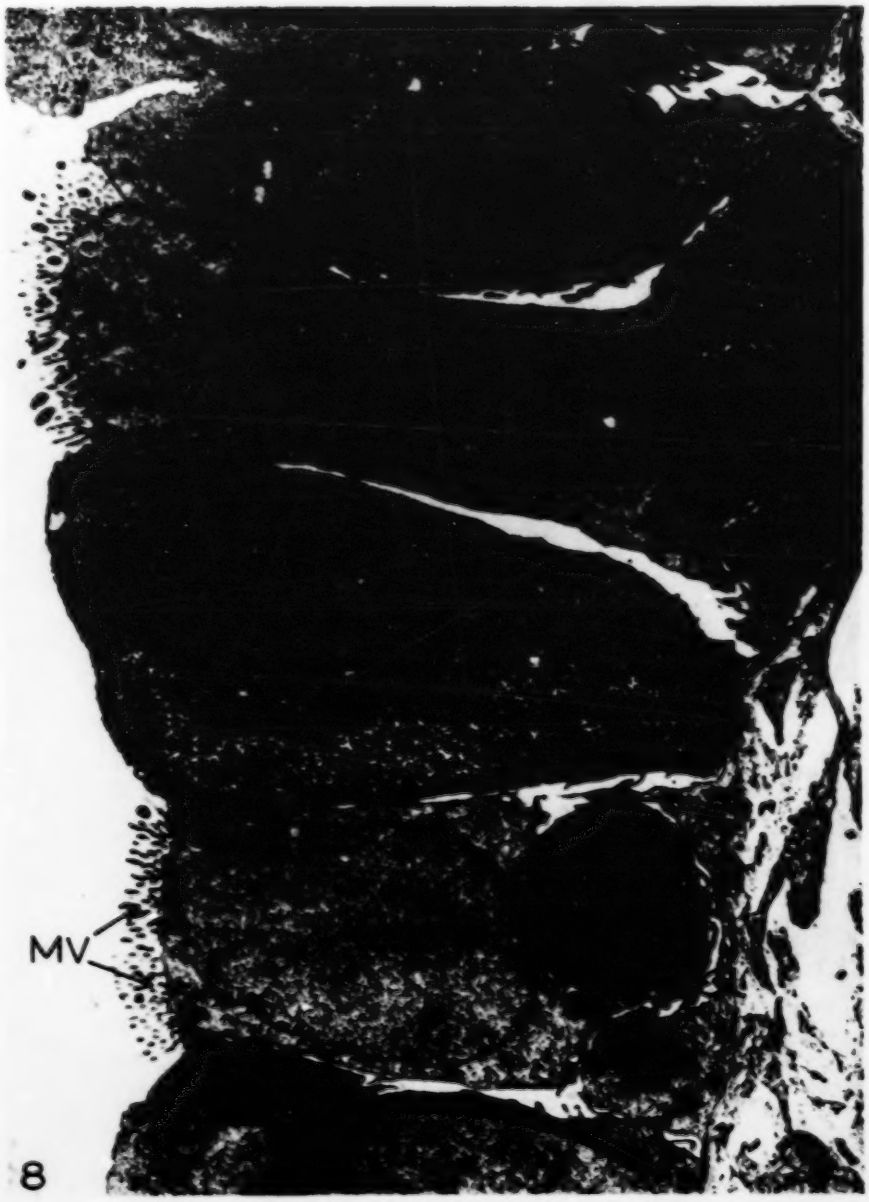

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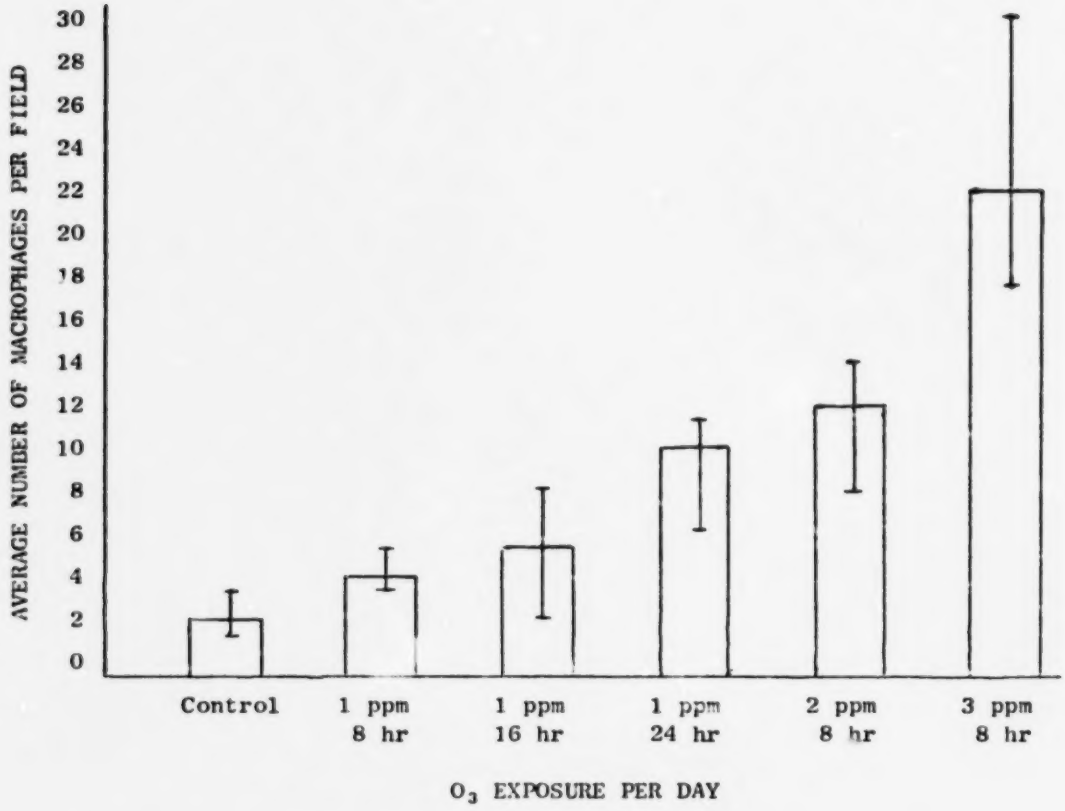
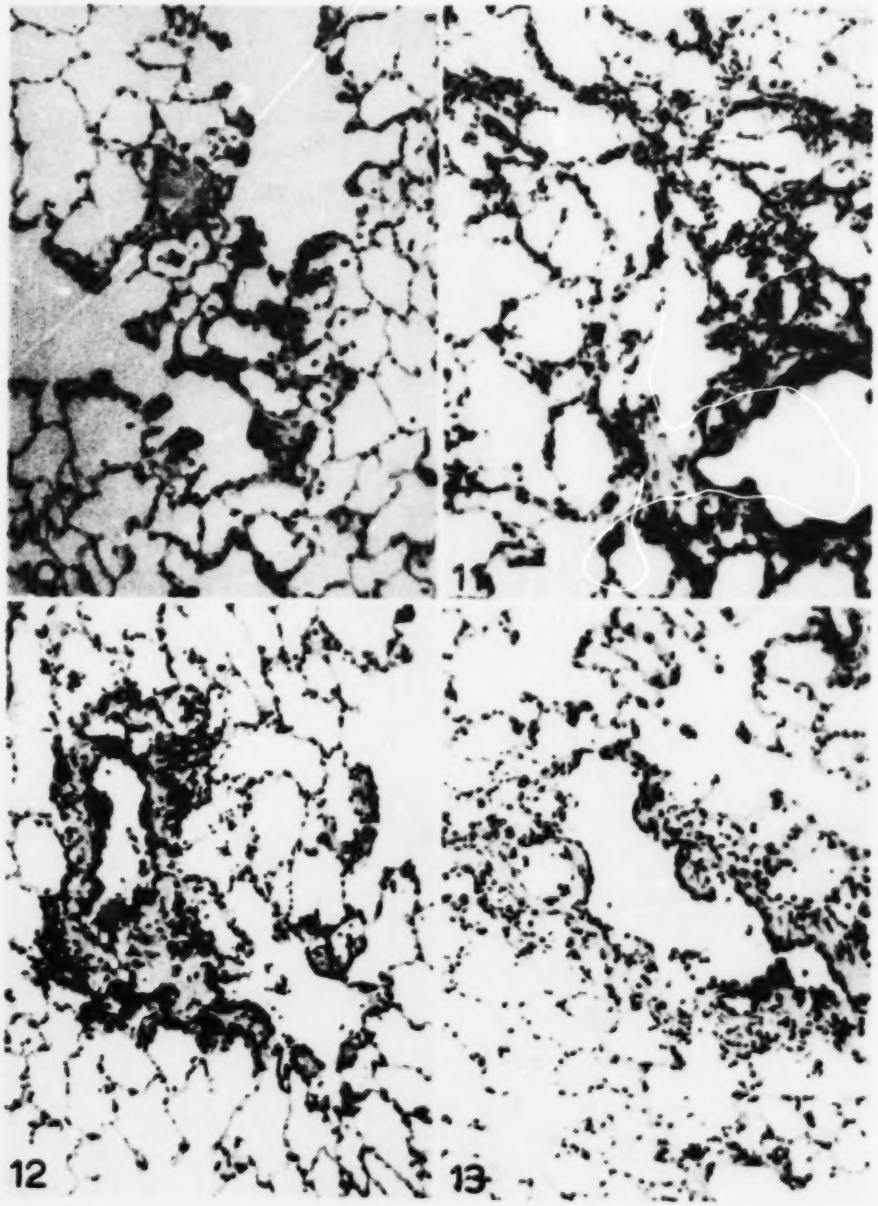


Figure 9. AVERAGE NUMBER OF MACROPHAGES ALONG PERIPHERAL AIRWAYS IN CONTROL AND EXPERIMENTAL LUNGS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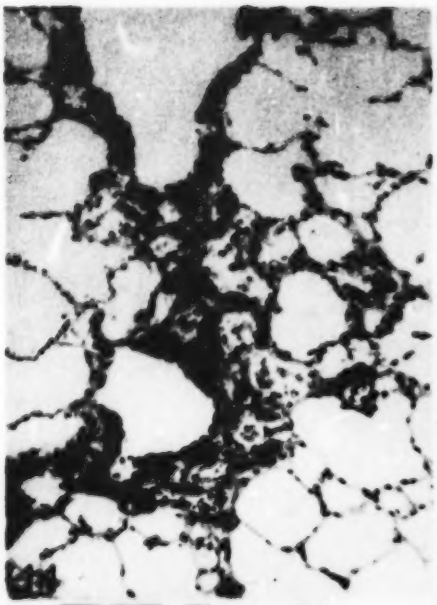

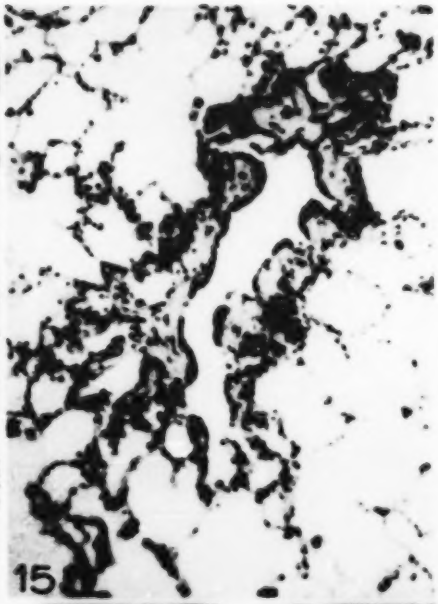
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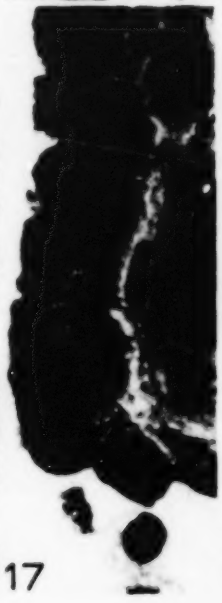
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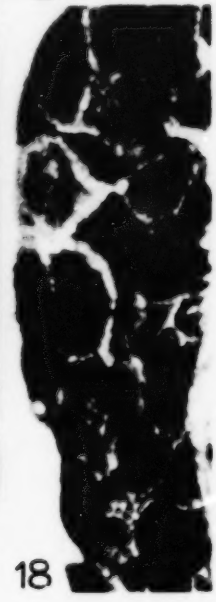
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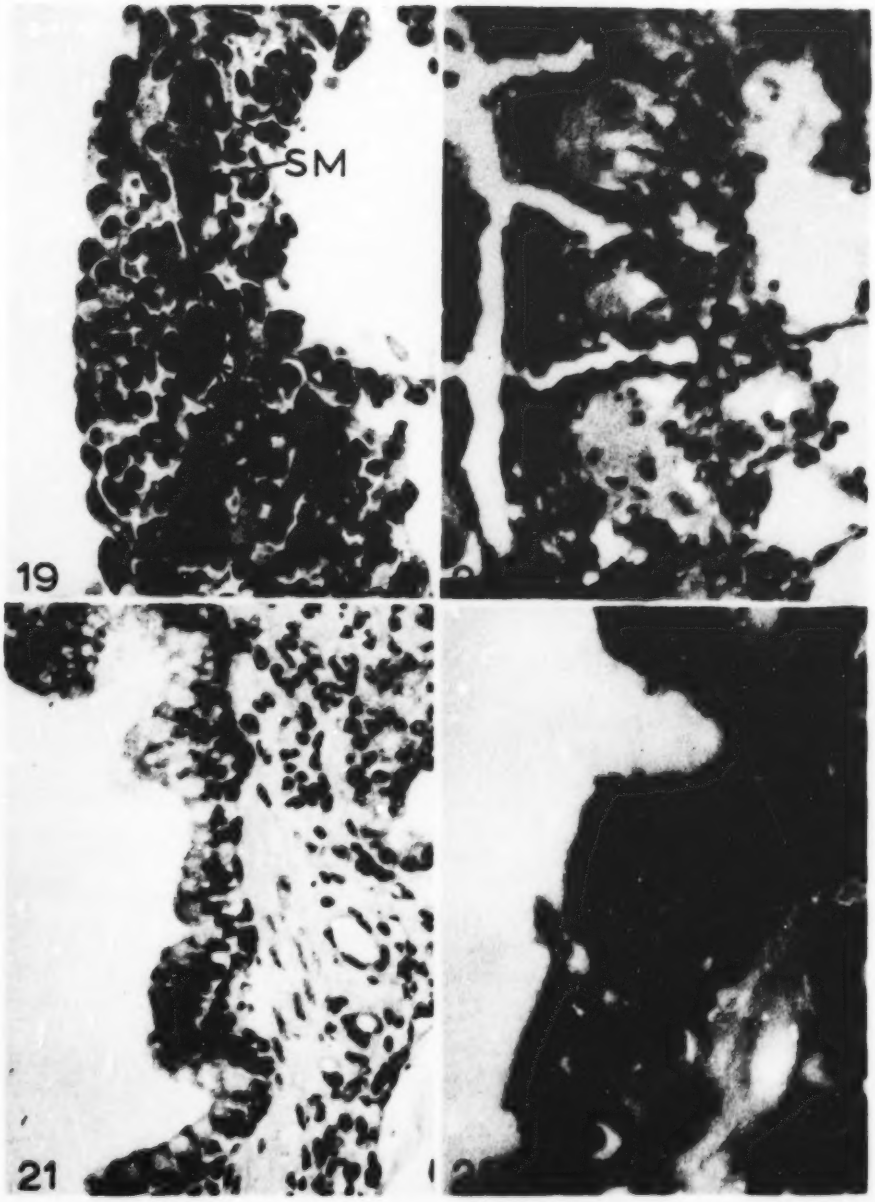
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